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The displacement of L-tryptophan and dipeptides from bovine albumin *in vitro* and from human plasma *in vivo* by antirheumatic drugs

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L-Tryptophan occurs in a protein-bound and an unbound form in serum from normal subjects. The amino-acid is displaced from its binding sites *in vitro* by salicylate, phenylbutazone, indomethacin, prednisolone, chloroquine and gold salts and is virtually absent in serum obtained from patients with rheumatoid arthritis receiving therapy with antirheumatic drugs. Some dipeptides bind to bovine albumin *in vitro* and are displaced by salicylate. All the drugs displace L-phenylalanyl-L-phenylalanine from its binding to normal human serum *in vitro*.

There have been many attempts to provide a unifying concept of the mode of action of the drugs commonly used in the treatment of rheumatism. These have included mechanisms based on stimulation of the anterior pituitary and adrenal cortex glands, on an interference with either the formation, release or action of suspected mediators of inflammation, on the abilities of the drugs to uncouple oxidative phosphorylation reactions and on a stabilizing effect of the drugs on lysosomal membranes thus preventing the release of hydrolase enzymes which can degrade joint cartilage. None of these theories adequately explains the clinical anti-inflammatory actions of the salicylates (Smith, 1966) or of the other antirheumatic remedies (Whitehouse, 1965; Domenjoz, 1966; Skidmore & Trnavsky, 1967; Houck & Forscher, 1968; Spector & Willoughby, 1968; Collier, 1969).

It has been shown (McArthur & Dawkins, 1969) that salicylate displaces L-tryptophan from its binding sites to human serum proteins. The present paper describes the effects of the commonly used antirheumatic drugs on the release of the amino-acid and certain dipeptides from their binding sites to bovine serum albumin and to human serum proteins.

MATERIALS AND METHODS

Materials

Pooled human serum was obtained from the National Transfusion Service, Sutton and individual samples of serum were obtained by venepuncture from 3 female and 2 male patients with rheumatoid arthritis under treatment at King's College Hospital. Bovine serum albumin (fraction V), amino-acids and dipeptides were obtained from the Sigma Chemical Company. The sodium salicylate was of British Pharmacopoeial standard, phenylbutazone was obtained from Geigy (U.K.), Ltd., as ampoules each containing 600 mg of the drug plus 30 mg of xylocaine in 3 ml, indomethacin powder

and prednisolone were obtained from Merck Sharp and Dohme Ltd., the latter being in the form of an injection containing 20 mg of prednisolone sodium phosphate, 2·5% (w/v) nicotinamide, 0·01 (w/v) sodium metabisulphite, 0·05% (w/v) sodium edetate and 0·5% (w/v) phenol in a volume of 2 ml. Chloroquine phosphate powder was obtained from ICI Ltd. and the gold salts from May & Baker Ltd. as an injection containing 100 mg of sodium aurothiomalate and 0·002% (w/v) phenylmercuric nitrate in 1 ml.

Measurement of tryptophan in human serum

The concentrations of tryptophan (total and free) were measured in pooled normal serum and in sera from patients with rheumatoid arthritis as described by McArthur & Dawkins (1969).

Tryptophan binding experiments

Samples (10 ml) of either pooled human serum or serum from patients with rheumatoid arthritis were applied to a 36 × 2·8 cm column containing 40 g of Sephadex G-25 medium. The column was washed with a solution containing 0·14M NaCl and 0·01M phosphate buffer, pH 7·4 (buffer A) and the first 65 ml of eluate were discarded. The next 35 ml, containing the proteins but free of amino-acids, was collected, placed in a 65 ml ultrafiltration cell equipped with a Diaflo PM 30 membrane (Amicon N.V., Holland), and the volume reduced to 10 ml. Buffer A (10 ml) was added to the protein solution inside the cell, the volume reduced to 10 ml and this process was repeated three times to ensure that all ultrafilterable ninhydrin-positive substances and as much as possible of the drugs had been removed. To the concentrated protein solution inside the cell was added 10 ml of a 0·1 mM solution of L-tryptophan in buffer A and 10 ml of ultrafiltrate collected. The first 7 ml of the ultrafiltrate were discarded to compensate for the "dead space" in the apparatus and the final 3 ml diluted with an appropriate quantity of buffer A and its content of free tryptophan estimated as described previously (McArthur & Dawkins, 1969). This procedure was repeated successively with 10 ml quantities of 0·3, 1·0 and 3·0 mM tryptophan and with 2·0 mM salicylate.

Binding of dipeptides to bovine albumin and displacement by salicylate

Samples (20 ml) of 3% (w/v) bovine albumin in buffer A were placed in a 65 ml ultrafiltration cell fitted with a Diaflo PM 30 membrane and the volume reduced to 10 ml. This procedure was repeated after the successive addition of 10 ml quantities of the phosphate buffer, and the following concentrations of either amino-acids or the dipeptides, 0·05 mM, 0·1 mM and either 0·5 mM (×2) or 0·3 mM and 1·0 mM, depending on the varying solubilities of the materials, followed by 2 mM salicylate. In each instance the first 7 ml of ultrafiltrate was discarded and the following 3 ml collected and analysed for ninhydrin-positive substances (McArthur & Dawkins, 1969), after appropriate dilution with the phosphate buffer.

Displacement of L-*tryptophan and* L-*phenylalanyl-*L-*phenylalanine from pooled human serum by antirheumatic drugs*

Samples (10 ml) of pooled human serum were cleared of amino-acids as described above except that the Sephadex column was washed with 0·01M phosphate buffer, pH 7·4, and not with buffer A. To the 10 ml of protein solution inside the 65 ml

ultrafiltration cell was added either 10 ml of 0·01M phosphate buffer, pH 7·4, or a 10 ml quantity of the buffer containing either 0·5 mM L-tryptophan or 0·5 mM L-phenylalanyl-L-phenylalanine and the volume reduced to 10 ml. The first 7 ml of ultrafiltrate was discarded and the final 3 ml used for analysis using ninhydrin and the Technicon autoanalyser (McArthur & Dawkins, 1969). This procedure was repeated successively with 10 ml quantities of either buffer alone or buffer containing 100, 200 or 400 μg/ml of each drug.

RESULTS

Tryptophan concentrations in human serum

The free and total tryptophan concentrations (μM/100 ml) in pooled normal serum were: 1·21 $\pm$ 0·22 and 6·36 $\pm$ 0·48 (s.d. n = 6) and in patients with rheumatoid arthritis were 0·33 $\pm$ 0·06 and 1·88 $\pm$ 0·28 (s.d. n = 5) respectively.

The results show that the concentrations of free and total tryptophan in sera obtained from five patients with active rheumatoid arthritis, who were receiving treatment with one or more antirheumatic drugs, were significantly reduced ($P < 0{\cdot}001$) compared to the corresponding concentrations in the normal serum.

Binding of tryptophan to serum from normal subjects and from patients

The patients with rheumatoid arthritis had all received aspirin plus either phenylbutazone, indomethacin or gold salts. The serum samples and pooled normal serum were exhaustively treated by ultrafiltration to remove any amino-acids and drugs present, then exposed to increasing concentrations of L-tryptophan followed by salicylate. Free and total tryptophan concentrations were determined. There was no difference between the results from the pooled normal serum and from the patients' sera for each concentration of the amino-acid but the subsequent displacement of the bound tryptophan by 2 mM salicylate was significantly greater ($P < 0{\cdot}01$) for the normal serum.

Binding of dipeptides to bovine albumin

L-Tryptophan was found to be the only amino-acid to bind to the bovine albumin and none of the following dipeptides; glycylglycine, glycyl-L-serine, L-valyl-L-leucine, glycyl-L-phenylalanine, L-phenylalanylglycine, glycyl-L-tryptophan and L-tryptophanylglycine showed measurable binding to the protein. In contrast, the dipeptides listed in Table 1 resembled tryptophan in their binding characteristics and were all displaced by 2 mM salicylate, a concentration of the drug which is attained and maintained during the treatment of rheumatoid arthritis. These results are too limited to draw any conclusions about possible structural requirements for binding with the obvious reservation that the presence of either two phenylalanyl, two tryptophanyl or one of each amino-acid residues in a peptide may be an important factor in the binding to albumin. L-Phenylalanyl-L-phenylalanine did not displace any of the amino-acid bound to bovine serum albumin.

Displacement of L-tryptophan and L-phenylalanyl-L-phenylalanine from human serum by antirheumatic drugs

Normal human serum, when cleared of amino-acids, binds tryptophan and phenylalanylphenylalanine. When the cleared sera, exposed to 0·5 mM tryptophan or the

Table 1. *Binding of dipeptides to bovine serum albumin.* Each value is given as the % of the total concentration of dipeptide present which is bound to serum albumin and represents the mean of two separate experiments. The results in the presence of salicylate are expressed in the same way except that the total concentrations of dipeptides present are in brackets. The results with L-tryptophan have also been included for comparison.

Dipeptide	Total concentration of dipeptide present (mM) 0·025	0·06	0·20	0·60	In presence of salicylate (2 mM)
L-Phenylalanyl-L-phenylalanine	85	83	75	68	27 (0·48)
L-Phenylalanyl-L-tryptophan ..	76	74	67	63	38 (0·54)
L-Tryptophanyl-L-phenylalanine	89	87	84	77	40 (0·59)
L-Tryptophanyl-L-tryptophan ..	99	95	92	88	71 (0·45)
L-Tryptophan	80	77	64	43	36 (0·47)

dipeptide, are ultrafiltered and additional quantities of buffer added, then the concentrations of unbound tryptophan or dipeptide in the successive ultrafiltrates are progressively reduced. In the presence of either phenylbutazone, indomethacin, prednisolone, chloroquine or gold the amounts of unbound tryptophan or dipeptide in the ultrafiltrates are greater than in the absence of the drugs (Fig. 1). These drugs therefore displace the protein-bound amino-acid and dipeptide, the effect becoming more pronounced as the serum plus bound substance becomes exposed to increasing concentrations of each drug. The results of preliminary experiments also indicated that the effect of gold salts becomes more pronounced if the cleared serum was incubated for 16 h at 4° with the sodium aurothiomalate before exposure to the

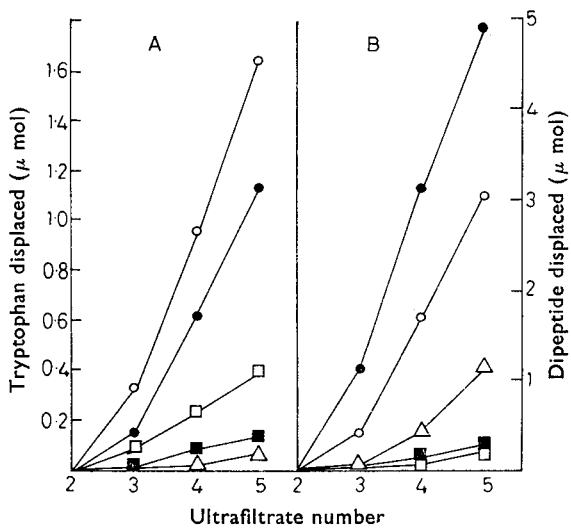

FIG. 1. Displacement of L-tryptophan and L-phenylalanyl-L-phenylalanine from human serum by antirheumatic drugs. The results have been plotted as the cumulative displacement of either the amino-acid or the dipeptide against the ultrafiltrate number. The second ultrafiltrate was obtained after the initial exposure of the cleared serum to 0·5 mM tryptophan or dipeptide, the third, fourth and fifth ultrafiltrates were obtained after the subsequent addition of 100, 200 or 400 μg/ml of the drugs. Each point represents the mean of two separate determinations. A, L-tryptophan; B, L-phenylalanyl-L-phenylalanine; ○, indomethacin; ●, phenylbutazone; □, chloroquine; ■, gold; △, prednisolone.

tryptophan and ultrafiltration. Salicylate has not been included in Fig. 1 since it had already been established (Table 1) that the drug displaced both tryptophan and phenylalanylphenylalanine from albumin.

DISCUSSION

L-Tryptophan is the only amino-acid bound to human serum albumin (McMenamy & Oncley, 1958). It is displaced by salicylates both *in vitro* (McArthur & Dawkins, 1969) and *in vivo* (Smith & Lakatos, 1971). The present work shows that protein-bound and unbound tryptophan may be readily measured in normal human serum but that their concentrations are significantly reduced in the serum of patients with rheumatoid arthritis receiving therapy with one or more antirheumatic drugs (Table 1). However, when the drugs are removed from the patients' sera, tryptophan binds to the proteins but is less easily displaced by salicylate than from normal serum. The other commonly used antirheumatic drugs, phenylbutazone, indomethacin, prednisolone, chloroquine and gold displace the amino-acid from its binding to human serum (Fig. 1).

The results in Table 1 show that certain dipeptides bind to bovine albumin and are displaced by a therapeutic concentration of salicylate. The binding of one of these dipeptides, L-phenylalanyl-L-phenylalanine, is affected by all the antirheumatic drugs (Fig. 1).

Although the slopes of the plots for the individual drugs in Fig. 1 show their relative potencies in displacing either tryptophan or phenylalanyl–phenylalanine from human serum *in vitro* this need not represent their antirheumatic activities since neither the amino-acid nor the dipeptide necessarily mediate clinical anti-inflammatory actions. They can only be interpreted as a qualitative guide that these antirheumatic drugs share a common biochemical action. It remains to be shown if drugs, which bind extensively to circulating proteins but which are devoid of clinical antirheumatic activity, lack the ability to displace either tryptophan or the dipeptide. However, it is possible that many drugs of this type could exert antirheumatic actions in man if they could be administered in sufficient dosage over long enough periods of time. This may apply to long-chain fatty acid anions, such as oleate, which have been shown (McMenamy & Orcley, 1958) to displace tryptophan from its binding sites to human albumin.

All the effective antirheumatic remedies have to be administered in divided doses over long periods of time, amounting to years for rheumatoid arthritis (Bluestone, 1970). The necessity for continued administration coupled with the relapses which occur when the drugs are stopped suggest that they support a natural defensive reaction against chronic inflammatory stimuli. Secondly, all the drugs bind to circulating proteins, particularly albumin. If given in therapeutic amounts only a relatively small fraction of the drugs exist in the unbound form. For example, salicylate is bound up to 90% at therapeutic concentrations. Toxicity, rather than clinical effectiveness, appears to be associated with the accumulation of unbound salicylate (Dawkins & Smith, 1971). Thus the protein-bound rather than the unbound forms of the drugs exert the chronic antirheumatic effects.

When the drugs bind to circulating albumin they displace other biologically active small molecules from their binding sites on serum proteins. The present work shows that this displacement extends to dipeptides which bind to human serum proteins. It is suggested that the unbound forms of some peptides may exert a

protective effect against the actions of mediators of chronic inflammatory insults. The binding of these hypothetical peptides to circulating proteins may be abnormally strong in patients with the rheumatic diseases because in such patients the circulating albumin possesses an abnormal amino acid composition (Denko, Purser & Johnson, 1970). The fraction of the peptides present in the free form would then be insufficient to exert a protective role. It is proposed that the antirheumatic drugs act by increasing the proportion of free peptides in the blood.

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